Intrahepatic upregulation of RhoA and Rho-kinase signaling contributes to increased hepatic vascular resistance in rats with secondary biliary cirrhosis

Inaugural-Dissertation

zur Erlangung des Doktorgrades

der Hohen Medizinischen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität

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Vorgelegt von

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INHALTSVERZEICHNIS

ZUSAMMENFASSUNG

Hintergrund: Bei der Leberzirrhose trägt ein erhöhter intrahepatischer Gefäss-Widerstand zur portalen Hypertension bei. Der RhoA/Rho-Kinase Signalweg ist einer der Hauptmechanismen, der zur Kontraktion glatter Gefässmuskulatur führt. Wir untersuchten, ob dieser Signalweg eine Rolle für den erhöhten intrahepatischen Durchblutungswiderstand bei Ratten mit sekundär biliärer Leberzirrhose spielt.

Methodik: Bei Ratten mit sekundär biliärer Leberzirrhose durch Gallengangsligatur (bile-duct ligation, BDL) und sham-operierten Kontrolltieren wurde über quantitative RT-PCR und Western-Blot Analyse die hepatische RhoA- und Rho-Kinase-Expression untersucht. Die Rho-Kinase Aktivität wurde als Phosphorylierung ihres Substrates, Moesin, bestimmt (Western-Blot Analyse, Immunohistochemie). In in situ perfundierten Lebern wurde der Effekt des Rho-Kinase Inhibitors Y-27632 auf den basalen und Methoxamin-stimulierten (alpha1-Adrenozeptor Agonist) Perfusionswiderstand untersucht. In anästhesierten Ratten wurde der hämodynamische Effekt einer intravenösen Applikation von Y-27632 untersucht (invasive Druckmessungen, kolorierte Mikrosphären).

Ergebnisse: Sowohl die mRNA- als auch die Protein-Expression von RhoA und Rho-Kinase waren in Lebern von BDL Ratten im Vergleich zu Lebern sham-operierter Kontrolltiere erhöht. Die Moesin-Phosphorylierung (Thr-558) war in Lebern zirrhotischer Ratten und auch von Patienten mit Alkohol-induzierter Leberzirrhose im Vergleich zu den zugehörigen nicht-zirrhotischen Kontrollen erhöht. Der Perfusionsdruck in situ perfundierter Lebern war bei BDL Ratten erhöht, und wurde bei BDL Ratten, nicht aber sham-operierten Ratten, durch Y-27632 gesenkt. Der Effekt von Y-27632 auf die Methoxamin-Hypersensitivität in situ perfundierter Lebern von BDL Ratten war stärker ausgeprägt als der Effekt auf die intrahepatische Methoxamin-Sensitivität sham-operierter Ratten. In vivo führte Y-27632 bei BDL Ratten, nicht aber bei sham-operierten Ratten, über eine Verminderung des intrahepatischen Widerstandes zu einer Senkung des Pfortaderdruckes.

Diskussion: Eine erhöhte Expression und nachfolgende Überaktivierung des RhoA/Rho-Kinase Signalweges trägt bei BDL Ratten zur Erhöhung des intrahepatischen Widerstandes, und so zur portalen Hypertension bei.

Keywords: cirrhosis; portal hypertension; intrahepatic vascular resistance; RhoA; Rho-kinase

INTRODUCTION

Increased resistance to portal blood flow is a primary factor in the pathophysiology of portal hypertension [1–4]. Anatomical abnormalities—such as narrowing of intrahepatic microvessels because of fibrosis—are a major cause of the increased resistance to portal flow. However, a dynamic component caused by an abnormally active contraction of the hepatic microvasculature plays an additional role in the development of increased intrahepatic resistance [5, 6]. This part of the intrahepatic resistance to portal flow is regulated by intrahepatic portal venules and hepatic stellate cells (HSCs) [7, 8]. Decreased formation and action of the vasodilator nitric oxide in the hepatic vascular bed supports the presence of portal hypertension [5, 9-16]. Furthermore, the intrahepatic resistance of cirrhotic livers shows hyperresponsiveness to vasoconstrictors such as noradrenaline (norepinephrine) and endothelin [5, 6, 17-22].

The RhoA/Rho-kinase pathway is essentially involved in vasoconstriction and the regulation of vascular tone [23-30]. The pathway is activated by contractile agonists through G-protein coupled vasopressor receptors (fig 1). These receptors activate the small monomeric GTPase, RhoA. Thereafter, RhoA activates Rho-kinase, which subsequently inhibits myosin-light-chain-phosphatase (MLC-phosphatase). Inhibition of MLC-phosphatase results in enhanced phosphorylation of MLCs and contraction.

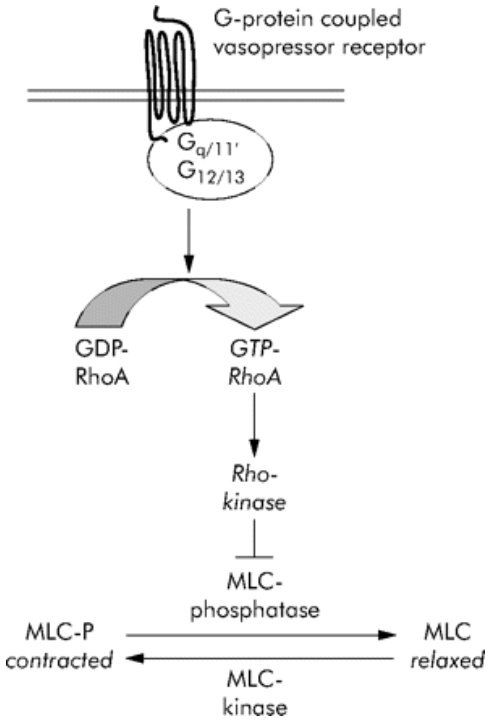

Figure 1 RhoA/Rho-kinase mediated vasoconstriction. The RhoA/Rho-kinase pathway is essentially involved in contraction of vascular smooth muscle and is linked to G-protein coupled receptors for vasoconstrictors. The initial event is the activation of the small monomeric GTPase RhoA by receptor associated heterotrimeric G-proteins containing α subunits of the $G\alpha_{q/11}$ and $G\alpha_{12/13}$ family. RhoA activation is associated with an exchange of GDP to GDP at the protein. GTP-RhoA subsequently activates Rho-kinase. Rho-kinase in turn phosphorylates and inhibits myosin-light-chain phosphatase. Inhibition of myosin-light-chain phosphatase results in enhanced phosphorylation of myosin-light-chains, which is the ultimate prerequisite for contraction of vascular smooth muscle. GDP, guanosine diphosphate; GTP, guanosine triphosphate.

Although it has been reported that RhoA is expressed in activated HSCs and that the RhoA/Rho-kinase pathway plays an important role in HSC activation and hepatic fibrogenesis [31-41], the actions of this pathway in the regulation of intrahepatic resistance to portal blood flow remain unknown. We therefore studied the role of the RhoA/Rho-kinase pathway in the intrahepatic vascular resistance of rats with secondary biliary cirrhosis and investigated whether it is involved in the pathogenesis of portal hypertension.

METHODS

Animals

Male Sprague-Dawley rats (180 to 200 g) were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained on standard chow on a 12 hour light/dark cycle. The rats were randomly divided into two groups. In one group, bile duct ligation was carried out as previously described [42]. Briefly, rats were anaesthetized with ketamine hydrochloride (100 mg/kg); the common bile duct was exposed by an upper abdominal midline incision of 1.5 cm and was ligated twice with 5-0 silk suture and resected between the ligatures; muscle and skin were sutured separately with 3-0 silk. The rats of the other group were sham operated and served as controls. These animals therefore experienced the same procedures except that the bile duct was manipulated but not ligated and sectioned.

The study was approved by the local committee for animal studies (administrative authority, Cologne, Germany, 50.203-Bn 15, 23/03).

Patients

Samples of liver tissue from patients with alcohol induced cirrhosis (n = 3) were obtained during liver transplantation. Non-tumour-bearing liver tissue obtained during resection of

liver malignancies served as normal control (n = 3). The use of human liver specimens was approved by the local ethics committee.

Western blot analysis

Samples of shock frozen livers were homogenized in a buffer containing 25 mM Tris/HCl, 5 mM ethylenediamine tetra-acetic acid, 10 μM phenylmethanesulphonyl fluoride, 1 mM benzamidine, and 10 μg/ml leupeptin. Protein determination of the homogenates was carried out with the Dc-Assay kit (Biorad, Munich, Germany). Thereafter, homogenates were diluted with sample buffer. Samples (20 μg of protein per lane) were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (15% gels for RhoA, 8% gels for Rho-kinase), and proteins were blotted on nitrocellulose membranes. The membranes were blocked, incubated with primary antibodies (RhoA 119 and Rock-2 H-85, Santa Cruz Biotechnology, Santa Cruz, California, USA) and thereafter with corresponding secondary peroxidase coupled antibodies (Calbiochem, San Diego, California, USA). Blots were developed with enhanced chemiluminescence (ECL, Amersham, UK). Intensities of the resulting bands on each blot were compared densitometrically with a FLA-3000 phosphoimager (Fuji-Film, Düsseldorf, Germany).

Quantitative real time reverse transcription polymerase chain reaction

RNA was isolated from 30 mg shock frozen liver tissue using the RNeasy-mini kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. RNA concentrations were measured spectrophotometrically at 260 nm. For each sample, 1 μg of total RNA was used. Before reverse transcription, samples were DNA digested with RQ1 RNase-free DNase (Promega, Madison, Wisconsin, USA). Reverse transcription was carried out using Moloney murine leukaemia virus (MMLV) reverse transcriptase (Invitrogen, Karlsruhe, Germany) and random primers (250 ng, Microsynth, Balgach, Switzerland). Control reactions did not contain reverse transcriptase. Primers and probes for real time reverse transcription polymerase chain reaction (RT-PCR) were designed using the Primer

Express Software (Applied Biosystems, Foster City, California, USA) and custom synthesised by Microsynth and Applied Biosystems, respectively. Sequences of the primers and probes are given in table 1. Primers and probes for the housekeeping gene (18SrRNA) were provided by Applied Biosystems as a ready-to-use mix. RT-PCR was carried out using the ABI 7700 sequence detector (Applied Biosystems). The PCR reaction was done in a volume of 25 μl containing 12.5 μl 2x TaqMan PCR master mix (Roche Molecular Systems, Mannheim, Germany/Applied Biosystems) and 2 μl cDNA (equivalent to 67 ng total RNA). The final concentrations of the primers and probes are given in table 1. 18SrRNA served as the endogenous control. The final concentrations were 100 nM for primers and 200 nM for the probe. The results are expressed as the number of cycles (C_T value) at which the fluorescence signal exceeded a defined threshold. The difference in C_T value of the target cDNA and the endogenous control are expressed as negative ΔC_T values ($-\Delta C_T$). Thus higher $-\Delta C_T$ values denote higher mRNA levels. The ΔC_T method was used for quantification of the results. For all target genes and 18SrRNA, validation experiments were carried out according to the manufacturer's guidelines. In these experiments, it was shown that the efficiencies of the RT-PCR for the target gene and the endogenous control were approximately equal. Thus the ΔC_T method is suitable for relative quantification.

Gene	Primer/probe sequence 5'-3' (forward/reverse/probe)	Primer/probe concentration (nM)
RhoA	GGCAGAGATATGGCAAACAGG,	300
	TCCGTCTTTGGTCTTTGCTGA,	300
	CACTCCATGTACCCAAAAGCGCCAAM	100
Rho-kinase	CCCGATCATCCCCTAGAACC,	300
	TTGGAGCAAGCTGTCGACTG,	300
	ACAAAACCAGTCCATTCGGCGGC	200

Table 1 Primers and probes used for quantitative reverse transcription polymerase chain reaction for RhoA and Rho-kinase

Assessment of Rho-kinase activity

Rho-kinase activity was assessed as phosphorylation of the endogenous Rho-kinase substrate, moesin, at thr-558 [43-48]. This was done by western blot analysis using a site specific and phosphospecific anti-moesin antibody (Santa Cruz Biotechnology). In parallel, total moesin was analysed using a non-phosphospecific antibody (Santa Cruz Biotechnology).

Immunohistochemistry

Immunohistochemical staining of liver sections was carried out using the indirect immunoperoxidase technique as previously described [49] with the exception that the incubation with the primary antibody (site specific and phosphospecific anti-moesin antibody) was prolonged (overnight), and a swine anti-rabbit antibody (Dako, Carpinteria, California, USA) was used as secondary antibody.

In situ liver perfusion

In situ liver perfusion was carried out in a recirculating system according to a previously described technique [9]. Briefly, rats were fasted overnight but allowed free access to water. Only cirrhotic rats with ascites were included in the study. After being anaesthetised with ketamine hydrochloride (60 mg/kg), the abdomen was opened and the bile duct was cannulated with a polyethyl tube to monitor bile flow. Loose ligatures were placed around portal vein, common hepatic artery, spleen vein, and posterior vena cava just cranially to the confluence of the right renal vein. A 500 U dose of heparin was injected into the posterior vena cava. The portal vein was cannulated with a 14-gauge Teflon catheter, initiating liver exsanguinations by infusion (30 ml/min) of Krebs-Henseleit solution containing heparin (2 U/ml) and oxygenated with carbogen (95% O_2, 5% CO_2). The posterior vena cava was immediately cut caudally to the loose ligature, allowing the perfusate to escape. Thereafter, the thorax was opened and the right atrium was cut. Another catheter was introduced in the right atrium and pushed forward to the inferior vena cava. Next, all ligatures were pulled tight. At a constant flow (30 ml/min), perfusion pressure was monitored continuously and recorded digitally on-line. The preparation was allowed to stabilize for 20 minutes without any procedure.

Viability and stability of liver perfusion preparation

The criteria for liver viability included gross appearance of the liver, stable perfusion, bile production >0.4 µl/min*g, and stable buffer pH (7.4±0.1) during the initial 20 minute stabilization period. If one of the viability criteria was not met, the experiment was discarded.

Effect of the α1 adrenoceptor agonist methoxamine on portal perfusion pressure

In one set of experiments, livers were initially perfused at a constant flow (30 ml/min) for a period of 20 minutes without any procedure in order to stabilize the entire system. Then

cumulative concentration–response curves with the α_1 adrenoceptor agonist methoxamine (0.1 μM to 100 μM) were obtained by addition of the agonist to the perfusate. Changes in perfusion pressure were expressed either as the absolute perfusion pressure after administration of methoxamine, or as change in perfusion pressure elicited by the given concentration of methoxamine in the perfusate (that is, perfusion pressure minus basal pressure).

Effect of the Rho-kinase inhibitor Y-27632 on methoxamine induced hepatic flow resistance

In another set of experiments, 10 minutes before addition of the first dose of methoxamine, Y-27632 was added to the perfusate in different concentrations (1, 10, and 30 μM). Thereafter, cumulative concentration–response curves for methoxamine were constructed as described above.

Haemodynamic studies

Haemodynamic studies were carried out under ketamine anaesthesia (60 mg/kg intravenously). This condition has been shown to approximate most closely the conscious state in terms of cardiac output and regional blood flow and has been used extensively to investigate the haemodynamic effects of portal pressure lowering drugs in animal models of portal hypertension [2, 42, 50, 51]. The left femoral artery and vein were cannulated with PE-50 catheters for measurement of arterial pressure and blood withdrawal, as well as for drug infusion. Median laparotomy was carried out and a PE-50 catheter was introduced into a small ileocaecal vein and advanced to the confluence of the portal and splenic vein for the measurement of portal pressure. Through the right carotid artery another PE-50 catheter was advanced into the left ventricle under pulse curve control. This catheter was used for microsphere application. The catheters in the femoral artery and the portal vein were connected to pressure transducers (Hugo Sachs Electronic, March-Hugstetten, Germany) for blood pressure measurement. The zero point was 1 cm above the operating table.

Regional blood flows were measured using the coloured microsphere method, as previously described [42, 52]. A reference sample was obtained for one minute at a rate of 0.65 ml/min using a continuous withdrawal pump (Hugo Sachs Electronic). Then 300 000 yellow microspheres (15 μm diameter, Triton Technologies, San Diego, USA) were suspended in 0.3 ml saline containing 0.05% Tween and injected into the left ventricle 10 seconds after the withdrawal pump had been started. Upon completion of the haemodynamic measurements the animals were killed and the lungs, liver, kidneys, stomach, intestine, pancreas, and spleen were resected. The tissues were weighed, minced with scissors, and digested by addition of 14 ml/g tissue of 4 M KOH with 2% Tween, and subsequent boiling for one hour. The blood reference sample was digested by the addition of 3.8 ml 5.3 M KOH and 0.5 ml Tween and subsequent boiling for one hour. The digested tissues and blood samples were vortexed and filtered using Whatman Nucleopore filters (Whatman International, Maidstone, UK). The colour of the filtered microspheres was dissolved in 0.2 ml N,N-dimethylformamide and the absorption was measured by spectrophotometry. Thereafter, the number of microspheres per organ and organ perfusion was calculated using software obtained from Triton Technologies.

Porto-systemic shunting (PSS) was estimated as previously described, after the injection of 150 000 blue microspheres in 0.3 ml saline containing 0.05% Tween into an ileocaecal vein within 30 seconds [42,53]. The tissue microsphere content was calculated as described for the measurement of organ blood flow. PSS was calculated as the number of microspheres in the lungx100 divided by the number of microspheres in lung and liver. Portal venous inflow (PVI) was calculated as the sum of the blood flows to stomach, spleen, intestines, pancreas, and mesentery. Collateral blood flow (ml/minx100 g) was estimated as PVIxPSS/100. Vascular resistances were calculated from the ratio between perfusion pressure and blood flow of each vascular territory.

Statistical analysis

Data are presented as means (SEM) with the indicated number (n) of experiments. Analysis of variance (ANOVA) followed by Bonferroni/Dunn or the Mann–Whitney U test was used for comparison between groups (StatView 5.0, SAS Institute, Cary, North Carolina, USA). Probability (p) values of <0.05 were considered statistically significant. For the analysis of the in situ liver perfusion studies with methoxamine, concentration–response curves were fitted by non-linear regression, using the computer program Prism® (Graph Pad Software Inc, San Diego, California, USA). E_{max} (maximum contraction) and pEC_{50} values (negative logarithm of the concentration producing a half maximum effect) were calculated from the fitted curves.

RESULTS

Hepatic expression of RhoA and Rho-kinase

Western blot analysis of whole liver homogenates showed a strong upregulation of RhoA and Rho-kinase protein levels in livers from BDL rats compared with sham operated rats (fig 2A). In parallel, as revealed by quantitative RT-PCR with mRNA isolated from whole liver homogenates, both RhoA and Rho-kinase mRNA were significantly raised in livers from sham operated and BDL rats (fig 2B).

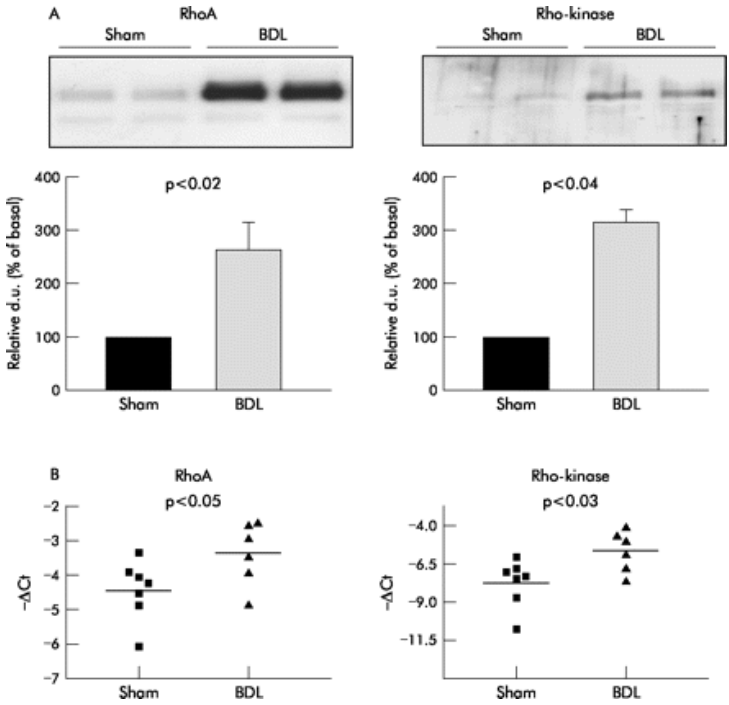

Figure 2 Hepatic RhoA and Rho-kinase expression in sham operated and bile duct ligated (BDL) rats. (A) Protein expression, western blot analysis. Shown are representative western blots of whole liver homogenates

and densitometric quantification of all experiments. Data are means with SEM, n = 8–10 for each group. (B) mRNA expression, data from quantitative reverse transcription polymerase chain reaction. Shown are mRNA levels in whole liver homogenates from both groups (sham n = 7, BDL n = 6). d.u., densitometric units.

Hepatic moesin phosphorylation

Phosphorylation of moesin—a marker for Rho-kinase activity [43-48] was greatly increased in livers of BDL rats (fig 3A). This difference was not associated with changes in total moesin, which was similar in both groups (fig 3A). As thr-558 of moesin is preferentially phosphorylated by Rho-kinase, these findings probably reflect an increased basal Rho-kinase activity in livers of BDL rats.

To localize the sites of intrahepatic moesin hyperphosphorylation in BDL rats, immunohistochemical investigations for phospho-moesin were undertaken in liver sections from sham operated and BDL rats. Phosphorylated moesin was highly present within the walls of the intrahepatic branches of portal venules and hepatic arteries (fig 4). The staining for phospho-moesin within these vessel walls was significantly stronger in BDL rats than in sham operated rats, and to some degree in the hepatic arteries (fig 4). In contrast, no phospho-moesin was detected in the remaining intrahepatic sites (hepatocytes or extracellular space), and only weak staining in the perisinusoidal cells (fig 4).

The phosphorylation state of moesin was also investigated by western blot analysis in human livers (whole liver homogenates from cirrhotic *v* non-cirrhotic patients). These experiments showed a clear trend towards raised phospho-moesin levels in livers from cirrhotic patients (alcohol induced cirrhosis) when compared with those from non-cirrhotic patients (fig 3B).

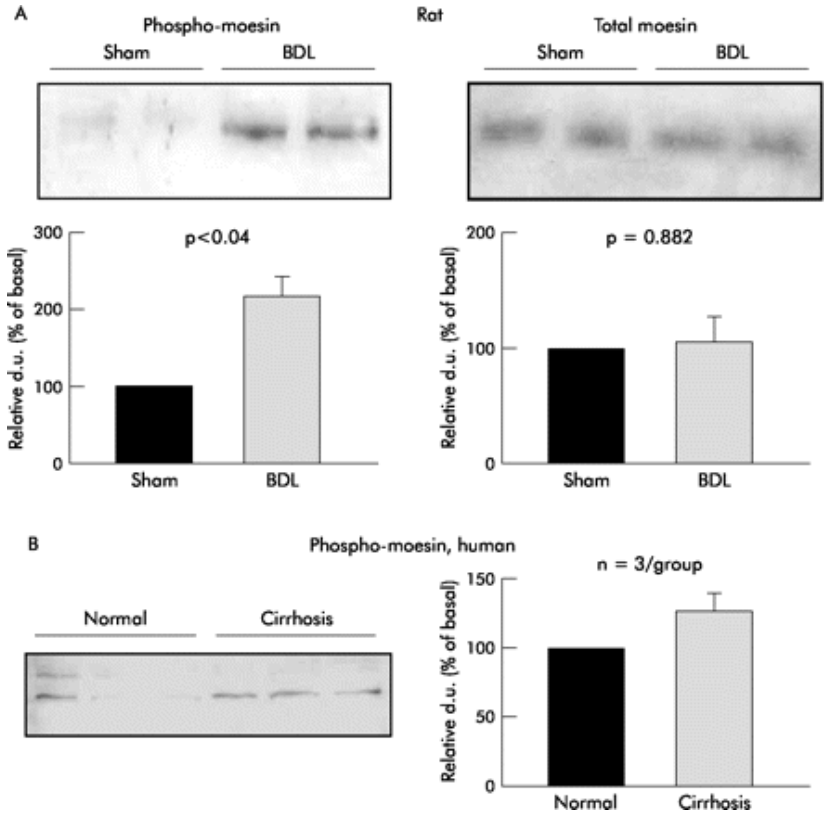

Figure 3 Total and phospho-moesin in whole liver homogenates in rat (A) and human (B) cirrhosis. Moesin is phosphorylated at threonine 558 by Rho-kinase. (A) Phospho-moesin and total moesin in livers from sham operated and bile duct ligated (BDL) rats (n = 5–6 for each group). (B) Phospho-moesin in livers from cirrhotic and non-cirrhotic patients (n = 3/group). Representative western blots of whole liver homogenates and densitometric quantification of all experiments are shown. Data are means with SEM; d.u., densitometric units.

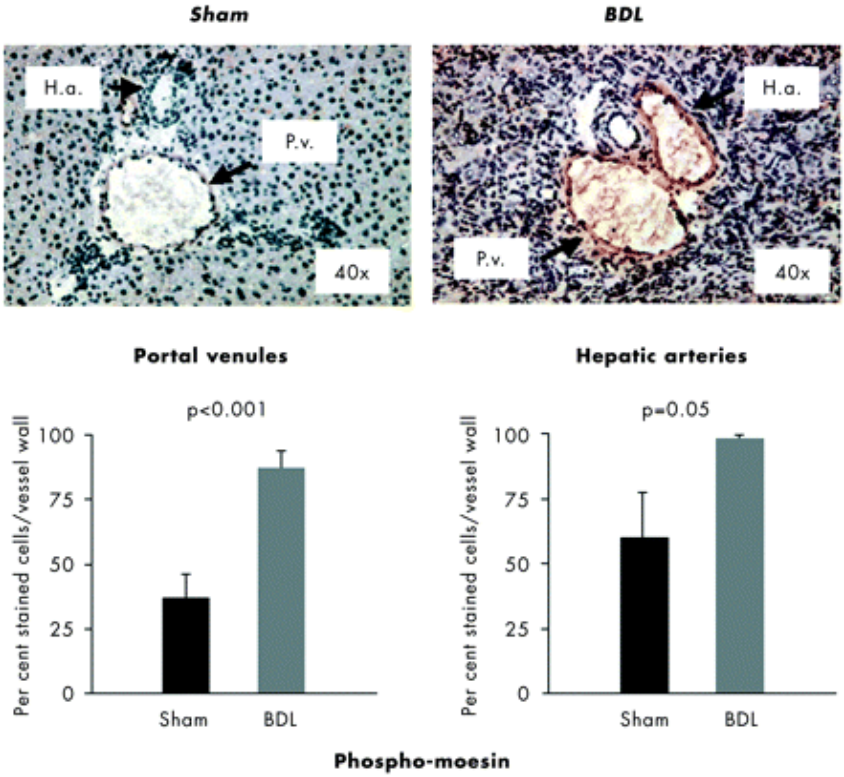

Figure 4 Immunohistochemical staining of liver sections from sham operated (n = 7) and bile duct ligated (BDL) rats (n = 7) for phospho-moesin. Representative experiments are shown, with quantification of the staining in the intrahepatic branches of the portal venules (P.v.) and hepatic arteries (H.a.). Data are means with SEM, n = 7 for each group (quantification was carried out in triplicate for each animal).

Basal intrahepatic resistance and modulation by Rho-kinase inhibition

At a constant flow, changes in perfusion pressure reflect changes in intrahepatic resistance. In BDL rats, the perfusion pressure of in situ perfused livers was on average increased threefold (fig 5A). In order to assess whether increased hepatic expression and activity of Rho-kinase is related to intrahepatic vascular resistance, we tested the effect of the Rho-kinase inhibitor Y-27632 on perfusion pressure. Y-27632 had no significant effects at any concentration on perfusion pressure in sham operated rats (fig 5B). In contrast, in BDL rats intrahepatic perfusion pressure was significantly reduced by 10 μM and 30 μM of Y-27632 (fig 5B).

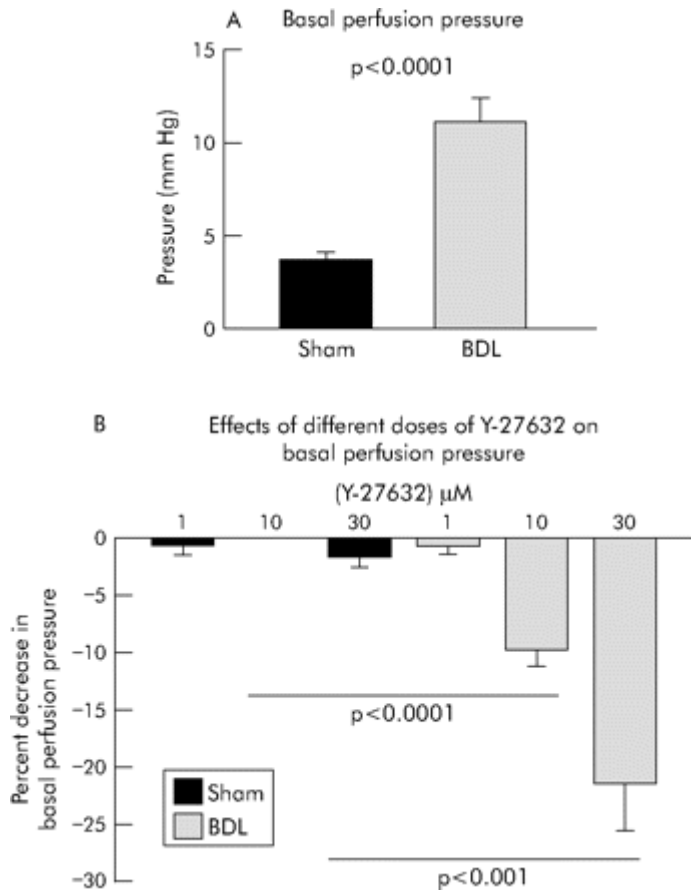

Figure 5. Basal perfusion pressure of in situ perfused livers (A), and effect of Rho-kinase inhibition with Y-27632 on basal perfusion pressure (B). Data are means with SEM, n = 9–10 in each group. BDL, bile duct ligated; sham, sham operated.

Effect of Rho-kinase inhibition on methoxamine induced increase in intrahepatic resistance

To investigate the role of the RhoA/Rho-kinase pathway in vasoconstrictor mediated regulation of intrahepatic microvascular tone, we studied the effect of Y-27632 on the methoxamine induced increase in intrahepatic perfusion pressure. As shown in fig 6A, the addition of methoxamine to the perfusate elicited dose dependent increases in perfusion pressure in both groups. The sensitivity of livers from BDL rats to methoxamine was significantly greater than in sham operated rats, as shown by the increase in EC_{50} (fig 6C, left columns). This underlines the well known hyperreactivity of the hepatic vascular resistance of cirrhotic livers to vasoconstrictors. However, the changes in perfusion pressure (P_{max}) elicited by the maximum concentration of methoxamine (100 μM) were similar in both groups (fig 6, A and D, left panels), although the perfusion pressures reached in BDL rats were higher than in sham operated rats, owing to the higher basal intrahepatic perfusion pressure.

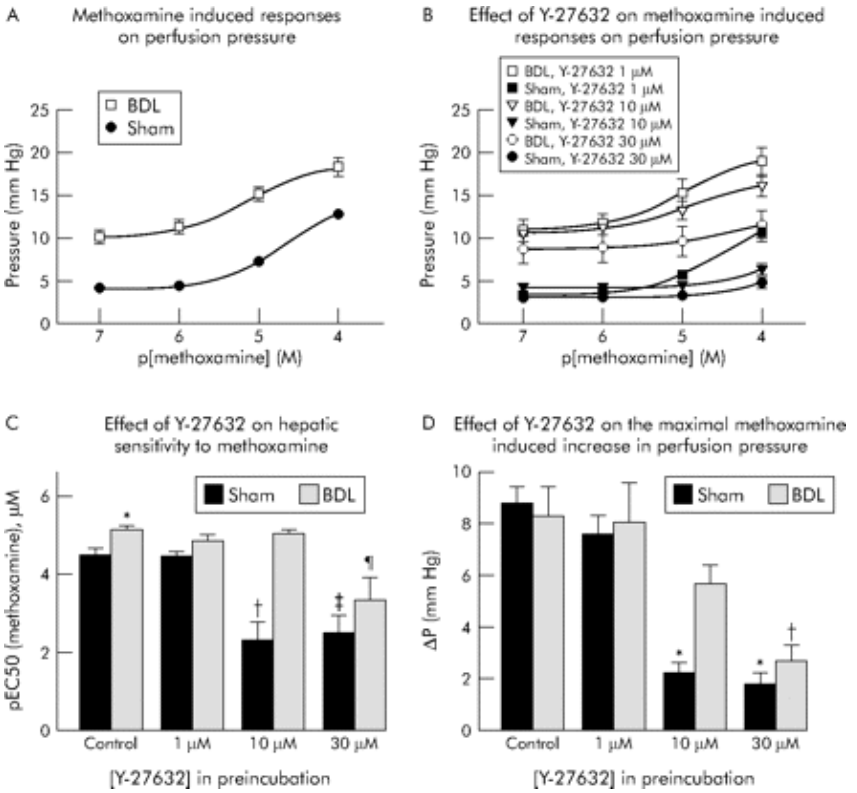

Figure 6 (A) Concentration–response curves for the effect of the α_1 adrenoceptor agonist methoxamine on the perfusion pressure of in situ perfused livers from sham operated (sham) and bile duct ligated (BDL) rats. (B) Effect of Rho-kinase inhibition with Y-27632 on the dose dependent methoxamine induced increase in perfusion pressure of in situ perfused livers. (C) Effect of Rho-kinase inhibition with Y-27632 on the methoxamine sensitivity of in situ perfused livers from sham operated and BDL rats. * p<0.002 *v* sham without Y-27632; † p<0.0004 *v* sham without Y-27632; ‡ p<0.0006 *v* sham without Y-27632; ¶ p<0.003 *v* BDL without Y-27632. (D) Reduction of methoxamine (100 μM) induced increase in perfusion pressure by Rho-kinase inhibition with Y-27632 in livers from sham operated and BDL rats perfused in situ. Data are means with SEM, n = 9–10 in each group. * p<0.0001 *v* sham without Y-27632; † p<0.0005 *v* BDL without Y-27632. p[methoxamine], negative decadic logarithm of a given concentration of methoxamine.

Next, we tested the effect of different concentrations of Y-27632 on the dose dependent methoxamine induced increase in intrahepatic resistance. The addition of 1 μM Y-27632 had no effect on the methoxamine induced increase in intrahepatic perfusion pressure in sham operated and BDL rats (fig 6, panels B and D). However, in sham operated rats, 10 μM and 30 μM Y-27632 significantly inhibited the P_{max} induced by 100 μM methoxamine (fig 6, panels A and D). In contrast, in BDL rats, only 30 μM Y-27632 significantly inhibited the methoxamine (100 μM) stimulated increase in perfusion pressure (fig 6D). Y-27632 10 μM and 30 μM significantly increased the EC_{50} to methoxamine of perfused livers from sham operated rats (fig 6C). By contrast, in BDL rats, only the 30 μM concentration of Y-27632 was able to affect the EC_{50} to methoxamine (fig 6C).

Haemodynamic in vivo effects of Rho-kinase inhibition

To obtain further insight into the regulation of portal pressure through the RhoA/Rho-kinase pathway in vivo, we studied the haemodynamic effects of bolus injection of different doses of Y-27632.

As expected, BDL rats had increased portal pressure, low arterial pressure, decreased splanchnic vascular resistance, increased intrahepatic resistance, and increased porto-systemic shunt flow. Bolus injection of Y-27632 (0.1 mg/kg) reduced portal pressure in BDL but not in sham operated rats (fig 7A). Interestingly, this was paralleled by a significant decrease in hepatic vascular resistance in BDL rats but not in sham operated rats (fig 8A). In contrast, splanchnic vascular resistance and hepatic arterial flow were unaffected in BDL rats, but reduced in sham operated rats after bolus injection of 0.1 mg/kg Y-27632 (fig 7, panels C and D). The shunt volume in BDL rats was significantly increased after Y-27632 (fig 8B). In both groups, the systemic administration of 0.1 mg/kg Y-27632 did not change mean arterial pressure (fig 7B). Bolus injection of Y-27632 in a dose of 1 mg/kg elicited large and sustained decreases in portal pressure in BDL rats but had no effect on portal pressure in sham operated rats (fig 7A). The reduction in portal pressure in response to the high dose of Y-27632 was associated with a further reduction in hepatic

vascular resistance (but also with further increases in portal venous inflow and shunt volume) in BDL rats, but not in sham operated rats (fig 8). In both groups, 1 mg/kg of Y-27632 induced large sustained decreases in mean arterial pressure (fig 7B), significant decreases in splanchnic vascular resistance, and significant increases in hepatic arterial flow (fig 7, panels C and D).

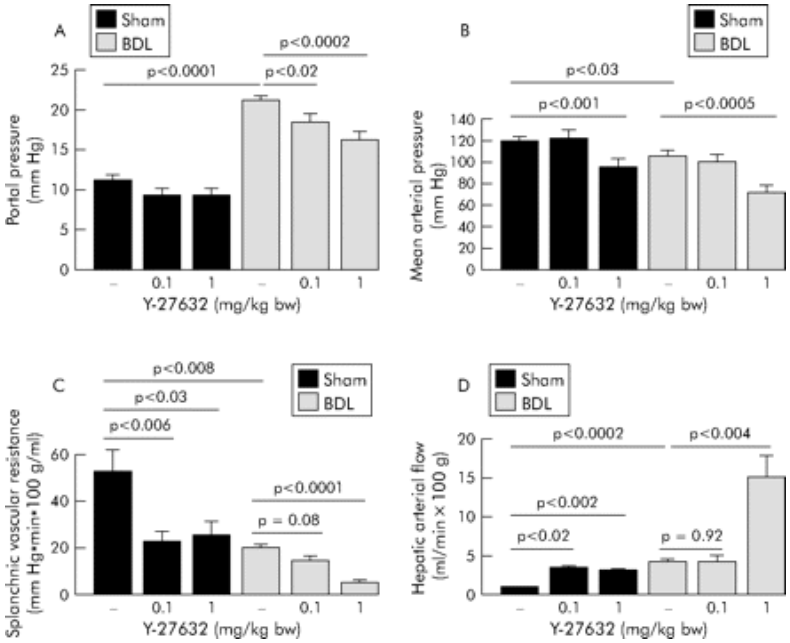

Figure 7 (A) Dose dependent in vivo effects of intravenous application of the Rho-kinase inhibitor Y-27632 on portal pressure in sham operated (sham) and bile duct ligated (BDL) rats, determined 45 minutes after bolus injection. (B) Dose dependent in vivo effects of intravenous application of the Rho-kinase inhibitor Y-27632 (0.1 and 1 mg/kg bw) on mean arterial pressure in sham operated and BDL rats, determined 45 minutes after bolus injection. Data are means with SEM, n = 11–27 in each group. (C) Dose dependent in vivo effects of intravenous application of the Rho-kinase inhibitor Y-27632 (0.1 and 1 mg/kg bw) on splanchnic vascular resistance in sham operated and BDL rats, determined 45 minutes after bolus injection. (D) Dose dependent in vivo effects of

intravenous application of the Rho-kinase inhibitor Y-27632 (0.1 and 1 mg/kg bw) on hepatic arterial flow in sham operated and BDL rats, determined 45 minutes after bolus injection. Data are means with SEM, n = 11–27 in each group. bw, body weight.

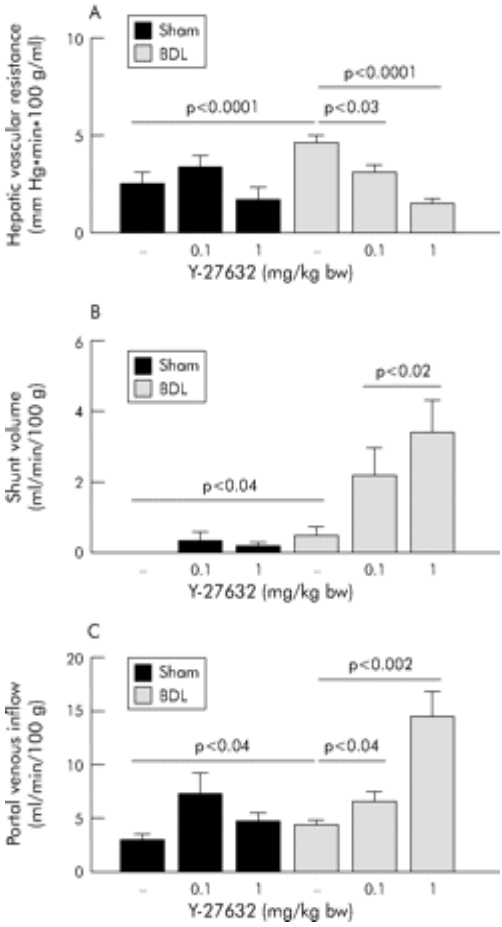

Figure 8 Dose dependent in vivo effects of intravenous application of the Rho-kinase inhibitor Y-27632 (0.1 and 1 mg/kg bw) on hepatic vascular resistance (A), shunt volume (B), and portal venous flow (C) in sham

operated (sham) and bile duct ligated (BDL) rats, determined 45 minutes after bolus injection. Data are means with SEM, n = 11–16 in each group.

DISCUSSION

The results of the present study provide evidence for the contribution of an abnormal increase in intrahepatic Rho-kinase signalling to the increased intrahepatic resistance and the increased sensitivity of the hepatic resistance to vasoconstrictors observed in rats with secondary biliary cirrhosis. An increased resistance of the intrahepatic microcirculation to portal flow contributes essentially to portal hypertension. Thus the intrahepatic regulation of liver blood flow by abnormal vasoconstrictor and vasodilator mediated signalling in cirrhosis has been studied widely [5.6.9-22]. To date, two mechanisms have been revealed which contribute functionally to the increased vascular tone in the cirrhotic liver. Increased intrahepatic resistance in cirrhosis is mediated by a decreased formation and action of the vasodilator nitric oxide (NO), and an increased sensitivity to vasoconstrictors (for example, α_1 adrenoceptor agonists and endothelin) [5.6.9-22]. This increased sensitivity to vasoconstrictors is not completely understood. Vasoconstrictor mediated signalling downstream of the level of G protein coupled vasopressor receptors could play a role. Therefore, we investigated the RhoA/Rho-kinase pathway in the vasoconstrictor mediated intrahepatic regulation of liver blood flow. It has been shown that this pathway is essentially involved in contraction of vascular smooth muscle [23-30]. Furthermore, a role for RhoA/Rho-kinase mediated signalling in activation and contraction of hepatic stellate cells as well as in hepatic fibrogenesis has also been demonstrated [31-41].

First, we investigated expression of RhoA and Rho-kinase in livers from sham operated and BDL rats. There was a strong upregulation of RhoA and Rho-kinase protein expression as well as mRNA expression in livers of rats with secondary biliary cirrhosis. The functional activity of Rho-kinase can be assessed as the phosphorylation state of its substrate moesin [43-48]. The hepatic upregulation of RhoA and Rho-kinase in cirrhotic rats indeed resulted in an increased moesin phosphorylation, reflecting an increased activity of Rho-kinase in

these animals. Most of the phosphorylated moesin found by immunohistochemical staining was localised to the walls of intrahepatic branches of portal venules and hepatic arteries, and to a lesser extent in perisinusoidal cells. Moesin phosphorylation in presinusoidal portal venules was highly increased in BDL rats. A similar increase in moesin phosphorylation was found by western blot analysis in liver homogenates from patients with alcohol induced cirrhosis. This indicates that these processes are not restricted to the animal model used here, but may probably be of clinical relevance.

To assess the functional relevance of the increased hepatic expression of RhoA and Rho-kinase and the subsequent increase in Rho-kinase activity for the hepatic vascular resistance of BDL rats, we tested the effect of the Rho-kinase inhibitor Y-27632 on the basal perfusion pressure of in situ perfused livers. Basal perfusion pressure at a constant flow (that is, the intrahepatic resistance to portal flow) was increased in BDL rats. Inhibition of Rho-kinase by Y-27632 reduced the basal perfusion pressure in BDL rats but not in sham operated rats. Thus the intrahepatic microcirculation in BDL rats was more susceptible to Rho-kinase inhibition than that of sham operated rats. As these findings were obtained under conditions excluding the influence of circulating vasoactive mediators, they possibly reflect an increased contribution of Rho-kinase to the increased basal vascular tone of the intrahepatic microvasculature in BDL rats.

Next, we studied the dose dependent changes in perfusion pressure after stimulation with the α_1 adrenoceptor agonist methoxamine in both groups. Livers from BDL rats were hypersensitive to methoxamine, as shown by the decreased EC_{50}. This increased sensitivity of livers from BDL rats to methoxamine shows the exaggerated response of cirrhotic livers to vasoconstrictors. To study the role of the RhoA/Rho-kinase pathway in the regulation of vasoconstrictor induced intrahepatic vascular tone, we examined the effect of different doses of Y-27632 on the methoxamine stimulated changes in perfusion pressure. In sham operated rats, Rho-kinase inhibition with Y-27632 at a concentration of 10 μM was already able to decrease the pEC_{50} for methoxamine. In contrast, at least 30 μM of Y-27632 was necessary to elicit the same effect in BDL rats. Furthermore, contractions

elicited by methoxamine were less susceptible to Rho-kinase inhibition in BDL rats than in sham operated rats.

These data permit several conclusions. First, contractile G-protein coupled receptors (for example, α_1 adrenoceptors) in the intrahepatic microvasculature are coupled to the RhoA/Rho-kinase pathway. Thus the RhoA/Rho-kinase pathway is indeed involved in the adrenergic regulation of intrahepatic vascular tone. Second, in BDL rats compared with sham operated rats, activation of the RhoA/Rho-kinase pathway in response to α_1 adrenergic stimulation of intrahepatic microcirculation is probably increased.

To investigate the role of the Rho-kinase signalling in intrahepatic resistance to liver blood flow and portal pressure in vivo, we studied the effects of systemic administration of the Rho-kinase inhibitor Y-27632 in anaesthetised rats. Portal pressure was dose dependently reduced in response to bolus injection of Y-27632 in cirrhotic but not in non-cirrhotic rats. Simultaneously, intrahepatic vascular resistance was decreased in BDL rats in response to Y-27632 but not in sham operated rats.

Taken together, in BDL rats Y-27632 decreased portal vascular resistance and hepatic vascular resistance after intravenous application; furthermore, Y-27632 reduced the intrahepatic perfusion pressure in the in situ perfused cirrhotic liver. These effects were much less pronounced in the sham operated rat. Because Y-27632 also efficiently reduced the perfusion pressure of in situ perfused livers, we assume that the portal pressure lowering effect of Y-27632 observed in vivo is at least partially mediated by intrahepatic actions of the inhibitor. However, it cannot be excluded that Y-27632 also directly acts at the portal vein itself. The decrease in hepatic vascular resistance of BDL rats was accompanied by an increased porto-systemic shunting. As microspheres were injected into the portal vein through the superior mesenteric vein to study porto-systemic shunting, we assume that Y-27632 decreased intrahepatic resistance at least in part by opening intrahepatic shunts. Hepatic arterial flow was increased in BDL rats despite an increase in moesin phosphorylation in intrahepatic hepatic arteries, suggesting further regulatory

pathways in these vessels counterbalancing an increased Rho-kinase activity. A contribution of hepatic arteries to the regulation of intrahepatic resistance is unclear. By contrast, portal venules are a principal site of regulation of intrahepatic resistance and portal pressures. This is emphasised by the pronounced effect of Y-27632 in livers perfused in situ.

The decrease in portal pressure was paralleled by a decrease in splanchnic vascular resistance. This decrease in splanchnic resistance might be explained by the vasodilating properties of Y-27632 on these vessels. Interestingly, the net decrease was greater in sham operated rats than in BDL rats, suggesting a reduced Rho-kinase activity in the splanchnic vasculature of the cirrhotic rats, which might contribute to the abnormally persistent vasodilatation of these vessels. The decrease in splanchnic vascular resistance increased portal tributary flow. This should normally increase portal pressure. However, in our BDL rats, portal pressure was decreased despite an increase in portal tributary blood flow, suggesting that the Y-27632 induced decrease in intrahepatic vascular resistance overcomes the increase in portal tributary flow. Again, this highlights the role of the hyperactivation of the RhoA/Rho-kinase pathway in the hepatic vascular bed for portal hypertension of BDL rats. It remains to be shown whether similar abnormalities in Rho-kinase signalling also contribute to the increased hepatic vascular resistance in other models of cirrhosis of the liver. As an increase in moesin phosphorylation was also found in livers from patients with alcohol induced cirrhosis, it seems possible that the Rho-kinase mediated increase in hepatic vascular resistance is not restricted to biliary cirrhosis in rats, but is rather a common feature of cirrhosis of the liver. Arterial pressure and splanchnic vascular resistance were also reduced by Y-27632. However, at the lower dose, Y-27632 reduced portal pressure in BDL rats without affecting arterial pressure. Thus most of the portal pressure lowering effect of Y-27632 seems to be mediated by decreasing intrahepatic resistance. Nevertheless, liver-specific drugs inhibiting the RhoA/Rho-kinase signaling preferentially in the cirrhotic liver should be developed before testing such drugs for pharmacological treatment of portal hypertension. Such Rho-kinase inhibiting drugs have the advantage that they also decrease hepatic fibrogenesis [38-41].

In summary, increased intrahepatic resistance in rats with secondary biliary cirrhosis is associated with an upregulation of RhoA and Rho-kinase signalling. Inhibition of this pathway in the liver can reduce portal pressure in rats with secondary biliary cirrhosis.

REFERENCES

1. **Hernandez-Guerra M**, Garcia-Pagan JC, Bosch J. Increased hepatic resistance. A new target in the pharmacological therapy of portal hypertension. *J Clin Gastroenterol* 2005;**39**:S131–7.
2. **Van de Casteele M**, Sägesser H, Zimmermann H, *et al.* Characterisation of portal hypertension models by microspheres in anaesthetised rats: a comparison of liver flow. *Pharmacol Ther* 2001;**90**:35–40.
3. **Menon KVN**, Kamath PS. Regional and systemic hemodynamic disturbances in cirrhosis. *Clin Liver Dis* 2001;**5**:617–27.
4. **Groszmann RJ**, Abraldes JG. Portal hypertension. From bench to bedside. *J Clin Gastroenterol* 2005;**39**:S125–30.
5. **Shah V**. Cellular and molecular basis of portal hypertension. *Clin Liver Dis* 2001;**5**:629–44.
6. **Rockey D**. The cellular pathogenesis of portal hypertension: stellate cell contractility, endothelin, and nitric oxide. *Hepatology* 1997;**25**:2–5.
7. **Reynaert H**, Thompson MG, Thomas T, *et al.* Hepatic stellate cells: role in microcirculation and pathophysiology of portal hypertension. *Gut* 2002;**50**:571–81.
8. **Rockey DC**. Hepatic blood flow regulation by stellate cells in normal and injured liver. *Semin Liver Dis* 2001;**21**:337–49.
9. **Wiest R**, Groszmann RJ. The paradox of nitric oxide in cirrhosis and portal hypertension: too much, not enough. *Hepatology* 2002;**35**:478–91.
10. **Shah V**, Haddad F, Garcia-Cardena G, *et al.* Liver sinusoidal endothelial cells are responsible for nitric oxide modulation of hepatic resistance. *J Clin Invest* 1997;**100**:2923–30.
11. **Dudenhoefer AA**, Loureiro-Silva MR, Cadelina GW, *et al.* Bioactivation of nitroglycerin and vasomotor response to nitric oxide are impaired in cirrhotic livers. *Hepatology* 2002;**36**:381–5.

12. **Gupta T**, Toruner M, Chung M, *et al.* Endothelial dysfunction and decreased production of nitric oxide in the intrahepatic microcirculation of cirrhotic rats. *Hepatology* 1998;**28**:926–31.
13. **Shah V**, Toruner M, Haddad F, *et al.* Impaired endothelial nitric oxide synthase activity associated with enhanced caveolin binding in experimental liver cirrhosis. *Gastroenterology* 1999;**117**:1222–8.
14. **Loureiro-Silva MR**, Cadelina GW, Groszmann RJ. Deficit in nitric oxide production in cirrhotic rat livers is located in the sinusoidal and postsinusoidal areas. *Am J Physiol Gastrointest Liver Physiol* 2002;**284**:G567–74.
15. **Yu Q**, Shao R, Qian H, *et al.* Gene transfer of the neuronal NO synthase isoform to cirrhotic rat liver ameliorates portal hypertension. *J Clin Invest* 2000;**105**:741–8.
16. **Rockey DC**, Chung JJ. Reduced nitric oxide production by endothelial cells in cirrhotic rat liver: endothelial dysfunction in portal hypertension. *Gastroenterology* 1998;**114**:344–51.
17. **Ghandi CR**, Sproat LA, Subbotin VM. Increased hepatic endothelin-1 levels and endothelin receptors density in cirrhotic rat livers. *Life Sci* 1996;**58**:55–62.
18. **Pinzani M**, Milani S, Franco R, *et al.* Endothelin-1 is overexpressed in human cirrhotic liver and exerts multiple effects on activated hepatic stellate cells. *Gastroenterology* 1996;**110**:534–48.
19. **Elliot AJ**, Vo LT, Grossmann VL, *et al.* Endothelin-induced vasoconstriction in isolated perfused liver preparations from normal and cirrhotic rats. *J Gastroenterol Hepatol* 1997;**12**:314–18.
20. **Grossmann HJ**, Grossmann VL, Bhathal PS. Enhanced vasoconstrictor response of the isolated perfused cirrhotic rat liver to humoral vasoconstrictior substances found in portal venous blood. *J Gastroenterol Hepatol* 1992;**7**:283–7.
21. **Graupera M**, Garcia-Pagan JC, Titos E, *et al.* 5-Lipoxygenase inhibition reduces intrahepatic vascular resistance of cirrhotic rat livers: a possible role of cysteinyl-leukotrienes. *Gastroenterology* 2002;**122**:387–93.

22. **Graupera M**, Garcia-Pagan JC, Abraldes JG, *et al.* Cyclooxygenase-derived products modulate the increased intrahepatic resistance of cirrhotic rat livers. *Hepatology* 2003;**37**:172–81.
23. **Somlyo AP**, Somlyo AV. Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol (Lond)* 2000;**522**:177–85.
24. **Uehata M**, Ishizaki T, Satoh H, *et al.* Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;**389**:990–4.
25. **Sakurada S**, Okamoto H, Takuwa N, *et al.* Rho activation in agonist-stimulated vascular smooth muscle. *Am J Physiol Cell Physiol* 2001;**281**:C571–8.
26. **Somlyo AP**, Somlyo AV. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G-proteins, kinases, and myosin phosphatase. *Physiol Rev* 2003;**83**:1325–58.
27. **Somlyo AP**, Wu X, Walker LA, *et al.* Pharmacomechanical coupling: the role of calcium, G-proteins, kinases and phosphatases. *Rev Physiol Biochem Pharmacol* 1999;**134**:201–34.
28. **Bishop AL**, Hall A. Rho GTPases and their effector proteins. *Biochem J* 2000;**348**:241–55.
29. **Etienne-Manneville S**, Hall A. Rho GTPases in cell biology. *Nature* 2002;**420**:629–635.
30. **Riento K**, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nature Rev Mol Cell Biol* 2003;**4**:446–56.
31. **Yanase M**, Ikeda H, Matsui A, *et al.* Lysophosphatidic acid enhances collagen gel contraction by hepatic stellate cells: association with rho-kinase. *Biochem Biophys Res Commun* 2000;**277**:72–8.
32. **Iwamoto H**, Nakamuta M, Tada S, *et al.* A p160ROCK-specific inhibitor, Y-27632, attenuates rat hepatic stellate cell growth. *J Hepatol* 2000;**32**:762–70.

33. **Lee JS**, Decker NK, Chatterjee S, *et al.* Mechanisms of nitric oxide interplay with Rho GTPase family members in modulation of actin membrane dynamics in pericytes and fibroblasts. *Am J Pathol* 2005;**166**:1861–70.
34. **Rombouts K**, Knittel T, Machesky L, *et al.* Actin filament formation, reorganization and migration are impaired in hepatic stellate cells under influence of trichostatin A, a histone deacetylase inhibitor. *J Hepatol* 2002;**37**:788–96.
35. **Kato M**, Iwamoto H, Higashi N, *et al.* Role of Rho small GTP binding protein in the regulation of actin cytoskeleton in hepatic stellate cells. *J Hepatol* 1999;**31**:91–9.
36. **Mizunuma K**, Ohdan H, Tashiro H, *et al.* Prevention of ischemia-induced hepatic microcirculatory disruption by inhibiting stellate cell contraction using rock inhibitor. *Transplantation* 2003;**75**:579–86.
37. **Kawada N**, Seki S, Kuroki T, *et al.* ROCK inhibitor Y-27632 attenuates stellate cell contraction and portal pressure increase induced by endothelin-1. *Biochem Biophys Res Commun* 1999;**266**:296–300.
38. **Muata T**, Arii S, Nakamura T, *et al.* Inhibitory effect of Y-27632, a ROCK inhibitor, on progression of rat liver fibrosis in association with inactivation of hepatic stellate cells. *Hepatology* 2001;**35**:474–81.
39. **Kanno K**, Tazuma S, Nishioka T, *et al.* Angiotensin II participates in hepatic inflammation and fibrosis through MCP-1 expression. *Dig Dis Sci* 2005;**50**:942–8.
40. **Tada S**, Iwamoto H, Nakatuma M, *et al.* A selective ROCK inhibitor, Y-27632, prevents dimethylnitrosamine-induced hepatic fibrosis in rats. *J Hepatol* 2001;**34**:529–36.
41. **Murata T**, Arii S, Mori A, *et al.* Therapeutic significance of Y-27632, a Rho-kinase inhibitor, on the established liver fibrosis. *J Surg Res* 2003;**114**:64–71.
42. **Heller J**, Shiozawa T, Trebicka J, *et al.* Acute haemodynamic effects of losartan in anaesthetized cirrhotic rats. *Eur J Clin Invest* 2003;**33**:1006–12.
43. **Fukuta Y**, Oshiro N, Kaibuchi K. Activation of moesin and adducin by Rho-kinase downstream of Rho. *Biophys Chem* 1999;**82**:139–47.

44. **Oshiro N**, Fukata Y, Kaibuchi K. Phosphorylation of moesin by rho-associated kinase (Rho-kinase) plays a crucial role in the formation of microvilli-like structures. *J Biol Chem* 1998;**273**:34663–6.

45. **Fukuta Y**, Kimura K, Oshiro N, *et al.* Association of the myosin-binding subunit of myosin phosphatase and moesin: dual regulation of moesin phosphorylation by Rho-associated kinase and myosin phosphatases. *J Cell Biol* 1998;**141**:409–18.

46. **Retzer M**, Essler M. Lysophosphatidic acid-induced platelet shape change proceeds via Rho/Rho kinase-mediated myosin light-chain and moesin phosphorylation. *Cell Signal* 2000;**12**:645–8.

47. **Shaw RJ**, Henry M, Solomon F, *et al.* RhoA-dependent phosphorylation and relocalization of ERM proteins into apical membrane/actin protrusions in fibroblasts. *Mol Cell Biol* 1998;**9**:403–19.

48. **Matsui T**, Maeda M, Doi Y, *et al.* Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J Cell Biol* 1998;**140**:647–57.

49. **Leifeld L**, Trautwein C, Dumoulin FL, *et al.* Enhanced Expression of CD80 (B7-1), and CD40 and Their Ligands CD28 and CD154 in Fulminant Hepatic Failure. *Am J Pathol* 1999;**154**:1711–20.

50. **Kojima H**, Yamao J, Tsujimoto T, *et al.* Mixed endothelin receptor antagonist, SB209670, decreases portal pressure in biliary cirrhotic rats in vivo by reducing portal venous system resistance. *J Hepatol* 2000;**32**:43–50.

51. **Seyde WC**, Longnecker DE. Anesthetic influence on regional hemodynamics in normal and hemorrhaged rats. *Anesthesiology* 1984;**61**:686–98.

52. **Hakkinen JP**, Miller MW, Smith AH, *et al.* Measurement of organ blood flow with coloured microspheres in the rat. *Cardiovasc Res* 1995;**29**:74–9.

53. **Geraghty JG**, Angerson WJ, Carter DC. Portal venous pressure and portosystemic shunting in experimental portal hypertension. Am J Phyiol 1989;257:G52–7.

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